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(54) Title: VARIANTS OF THE MAJOR ALLERGEN Par j 2 OF *Parietaria judaica*

(57) Abstract: Hypoallergenic variants of the major allergen Par j 2 of Parietaria judaica plants and the uses thereof in the therapy of allergicdiseases.

VARIANTS OF THE MAJOR ALLERGEN Par j 2 OF *Parietaria judaica*

The present invention relates to novel variants of an allergen of the pollen of plants of the species Parietaria judaica.

More particularly, the present invention relates to the amino acidic sequences of hypoallergenic variants of the allergen Par j 2, obtained by site-specific mutagenesis of the nucleotidic sequence encoding said allergen. The hypoallergenic variants can be used in the specific immunotherapy of allergic pathologies caused by Parietaria judaica pollen.

BACKGROUND OF THE INVENTION

Allergies are caused by a disfunction of the immune system, which reacts producing antibodies of the class IgE against proteins, mainly contained in pollens, mites, epithelia, and some foodstuff, which proteins are per se innocuous.

Recent estimations indicate that above 10% of population in Western countries suffers from this disease, which in time may induce worsening of symptoms (e.g. appearance of asthma) and sensitization to other allergens, thus making the choice of therapy more complicated.

Specific immunotherapy (SIT), contrary to pharmacological therapy, is the only kind of etiological treatment of allergic pathologies capable of favourably affecting some immunological parameters which are at the basis of the disease.

SIT consists in the administration of increasing doses of standardized extracts (vaccines), obtained starting from the substance which is the cause of the disease.

This way, immunological tolerance to said substance gradually increases in the patient, accompanied by disappearance of the allergic symptoms.

The risk of eliciting even serious side effects (1), although remarkably

reduced with the use of either slow-release vaccines or vaccines administered through alternative route to the injective one, has however restricted the use of SIT in the therapy of allergic diseases.

In recent years, attention has been focused on the development of effective, safer vaccines. In particular, an important target is the development of vaccines consisting of mutagenized recombinant proteins, i.e. hypoallergenic variants capable of favourably affecting the natural progress of the disease without causing undesired side effects (2).

Parietaria pollen is one of the most important causes of allergy in the Mediterranean area. The two main allergens of this pollen, Par j 1 (whose nucleotide sequence is identified in GenBank under the accession code AC X77414) and Par j 2 (AC X95865), are proteins of approximately 12 kD molecular weight with sequence and functional partial homology (3, 4, 5).

Detailed disclosure of the invention

It now has been found that the allergenic effect of Par j 2 (GenBank, AC X95865) may be decreased by changing its amino acid sequence in at least one of the positions n. 19, 23, 27, 35, 41, 46, 73 and 78, in which a Lys residue is present. "Change" herein means substituting one or more residues in the specified positions preferably with neutral or polar amino acids, or deleting one or more Lys residues present in the natural form, or simultaneously substituting and deleting two or more residues.

The following mutations by substitution, are preferred: Gln19, Ala23, Ala27, Gly35, Ala41, Ala46, Gly73 and Ser78, wherein the number means the position of the amino acidic residue in the sequence. More preferred are the variants in which the eight substitutions indicated above (SEQ ID N. 2) or, alternatively, the 5 substitutions Gln19, Ala23, Ala27, Ala41 and Ala46 (SEQ ID N. 3), are at the same time present.

The invention further comprises an immunologically active peptide

deriving from the amino acidic sequence of Par j 2 and containing at least one of the substitutions/deletions described above.

In a further aspect, the invention is directed to a nucleic acid molecule encoding for a protein variant of Par j 2 or for a peptide derived therefrom.

The sequence variants according to the invention can easily be prepared starting from cDNA of the allergen Par j 2 mature form, which does not include the region coding the signal peptide, suitably mutagenized at the desired positions.

The cDNA sequence coding the preferred variant corresponding to SEQ ID N. 2, is reported in SEQ ID N. 1.

The cDNA of SEQ ID N. 1 was expressed in Escherichia coli cells. The produced recombinant protein has reduced reactivity to IgEs of serum from subjects allergic to Parietaria judaica pollen. In particular, Western blotting tests proved that approximately 75% (10/13) of sera immunoreacting with the normal allergen have reduced reactivity with the mutagenized variant corresponding to SEQ ID N. 2 [Fig. 1]. This reactivity decreases by more than 80% on the average, as determined by ELISA assay [Fig. 2, mut 8]. On the other hand, the variant obtained by introducing the residues Gln19, Ala23, Ala27, Ala41 and Ala46 showed reduced allergenicity in half the tested sera [Fig. 1] and said reduction is 50% on the average [Fig. 2, mut 5]. Finally, the variant of the allergen Par j 2 with the 3 substitutions Gln19, Ala23 and Ala27 shows reduction of the IgE binding of about 20% [Fig. 2, mut 3].

The invention further relates to an expression vector comprising a nucleic acid molecule coding for any one of the hypoallergenic variants defined above.

Said vector can be a plasmid, cosmid, virus, bacteriophage or any other vector commonly used in genetic engineering, and can include, in addition to the nucleic acid molecule of the invention, eukaryotic or prokaryotic elements

for the control of the expression, such as regulatory sequences for the initiation and the termination of the transcription, enhancers, promoters, signal sequences and the like.

Moreover, the invention comprises a prokaryotic or eukaryotic host cell transformed into or transfected with the vector of the invention. In principle, prokaryotic cells such as Escherichia coli or Bacillus subtilis, or eukaryotic cells such as Saccharomyces cerevisiae will be used for cloning the vector and expressing the cDNA.

The protein variants of the invention can be produced either as such or as fusion proteins.

Thanks to the reduced IgE reactivity, said variants may be used for therapeutical purposes in the preparation of vaccines to be used in the immunotherapy of allergies to Parietaria judaica pollen.

A further aspect of the invention relates therefore to a pharmaceutical composition comprising an effective amount of the hypoallergenic variant of the invention, optionally in combination with other natural or modified allergens of Parietaria judaica, together with pharmaceutically acceptable excipients.

In a preferred embodiment, said pharmaceutical composition is a vaccine for use in the prophylactic or therapeutical treatment of allergic diseases, such as bronchial asthma, allergic rhinitis, allergic dermatitis, allergic conjunctivitis. Vaccination principles and practice are well known to those skilled in the art and are described, for example, in (7) and (8).

The following examples illustrate the invention in greater detail.

The methods used in the following examples, if not otherwise specified, are those described by Sambrook, Fritsch ET Maniatis "Molecular cloning. A laboratory manual" II Ed. vol. 1-2-3 CSH Lab Press 1989.

Example 1 - Site-specific mutagenesis of the cDNA coding for the allergen Par j 2

The site-specific mutagenesis of the cDNA coding for the allergen Par j 2 is carried out by PCR amplification (Polymerase Chain Reaction) of the same cDNA cloned in a prokaryotic vector (pBluescript).

The oligonucleotides used as primer for the PCR reaction have the required substitutions of bases. For each mutagenesis, a complementary pair of said oligonucleotides has been used, which bind to corresponding regions of the two DNA strands. After amplification, the original, unchanged template is selectively degraded by enzymatic digestion catalyzed by the restriction enzyme Dpn I. Escherichia coli cells are then transformed with the mutagenized molecules. Clones obtained from single bacterial colonies are sequenced according to the Sanger method to verify the correct modification of the bases and the absence of cDNA aspecific mutations.

Example 2 - Production of the protein Par j 2 and of the variants thereof

Normal cDNA from Par j 2 and mutagenized cDNA, after cloning in an expression vector (pCALn - Stratagene), are expressed in Escherichia coli cells according to standard protocols. Cells are collected by centrifugation and resuspended in PBS buffer. The recombinant proteins are isolated after lysis of the bacterial cells by sonication and removal of cell particulates by centrifugation. Proteins are purified from supernatant by affinity chromatography, using columns wherein the matrix is bonded to the calmodulin protein, which interacts with the CBP portion (Calmodulin Binding Protein) fused to the allergen.

Example 3 - Western blotting assay of the allergenicity of the Par j 2 variants

Equal amounts of the recombinant allergen and of the mutagenized variant are analyzed by electrophoresis on polyacrylamide gel and subsequent transfer onto nitro-cellulose membrane by electroblotting, according to the

technique described by Towbin (6).

The membrane is incubated for an hour in TBS containing 5% of powder milk (saturation buffer) then overnight with single sera from patients allergic to Parietaria (RAST 3+ and 4+). After three washings with TBS containing 0.05% Tween-20, IgE antibodies bound to the membrane are detected by incubation for an hour with anti-human IgE peroxidase-conjugated antiserum and, after further washings, with the detection system based on the use of a DAB (diaminobenzidine) solution containing H_2O_2 as substrate for the peroxidase.

Example 4 - ELISA assay for the reactivity to IgE of the Par j 2 variants

Equal amounts (0.2 μg) of normal allergen and of its mutagenized variants, in carbonate/bicarbonate 50 mM buffer, pH 9,6, are adsorbed on wells of polystyrene plates for ELISA tests by incubation at 4°C for 16 hours. The antigens are then washed with washing solution (60 mM phosphate buffer pH 6,5 containing 0.05% Tween-20) and the free sites are saturated with diluent solution (25% horse serum, EDTA 1 mM, 0.05% Tween 20, 0.01% Thiomersal in phosphate buffer 150 mM pH 7,4). Serial dilutions of human serum pools with RAST 4+ reactivity are prepared in a 1:2 ratio in diluent buffer. Equal amounts (100 μl) of the various serum dilutions are added to each sample and incubated at 25°C for 2 hours. After three washings, the anti-human IgE peroxidase conjugated antiserum diluted 1:1500 in diluent buffer is added, and incubated at 25°C for 1.5 hours. After three washings, the colorimetric reaction is developed by addition of 100 μl of Ultra Blu reagent (Intergen, Milford, MA) and incubation for 15 minutes at 25°C. The reaction is stopped by addition of 100 μl of 1N HCl and evaluated at 450 nm with a spectrophotometer.

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CLAIMS

1. A variant of the natural form of the protein Par j 2 wherein at least one of the Lys residues present at the positions 19, 23, 27, 35, 41, 46, 73 and 78 is substituted and/or deleted.

2 A variant as claimed in claim 1, wherein said residues are substituted with neutral or polar amino acids.

3. A variant as claimed in claims 1-2, wherein the substitutions are selected from Gln19, Ala23, Ala27, Gly35, Ala41, Ala46, Gly73 and Ser78.

4. A variant as claimed in claims 1-3, having sequence SEQ ID N. 2.

5. A variant as claimed in claims 1-3, having the substitutions Gln19, Ala23, Ala27, Ala41 and Ala46 (SEQ ID N. 3).

6. A peptide comprising an immunologically active part of a variant of claims 1-5, wherein at least one substitution/deletion as claimed in claim 1 is present.

7. A nucleic acid molecule coding for a protein variant as claimed in claims 1-5 or for a peptide as claimed in claim 6.

8. A nucleic acid molecule as claimed in claim 7, of sequence SEQ ID N. 1.

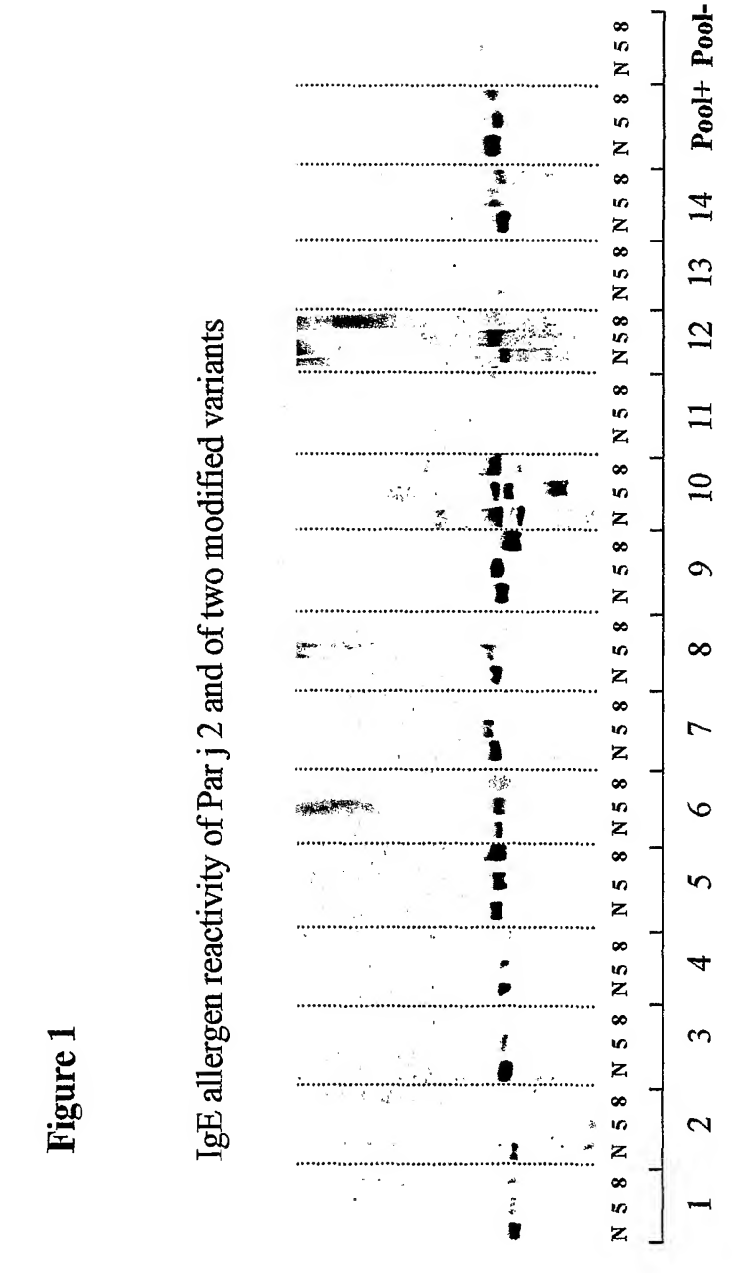
9. A vector comprising the nucleic acid molecule of claims 7-8.

10. A host cell transduced with the vector of claim 9.

11. A pharmaceutical composition comprising an effective amount of a protein variant as claimed in claims 1-5 or of a peptide as claimed in claim 6 together with pharmaceutically acceptable excipients.

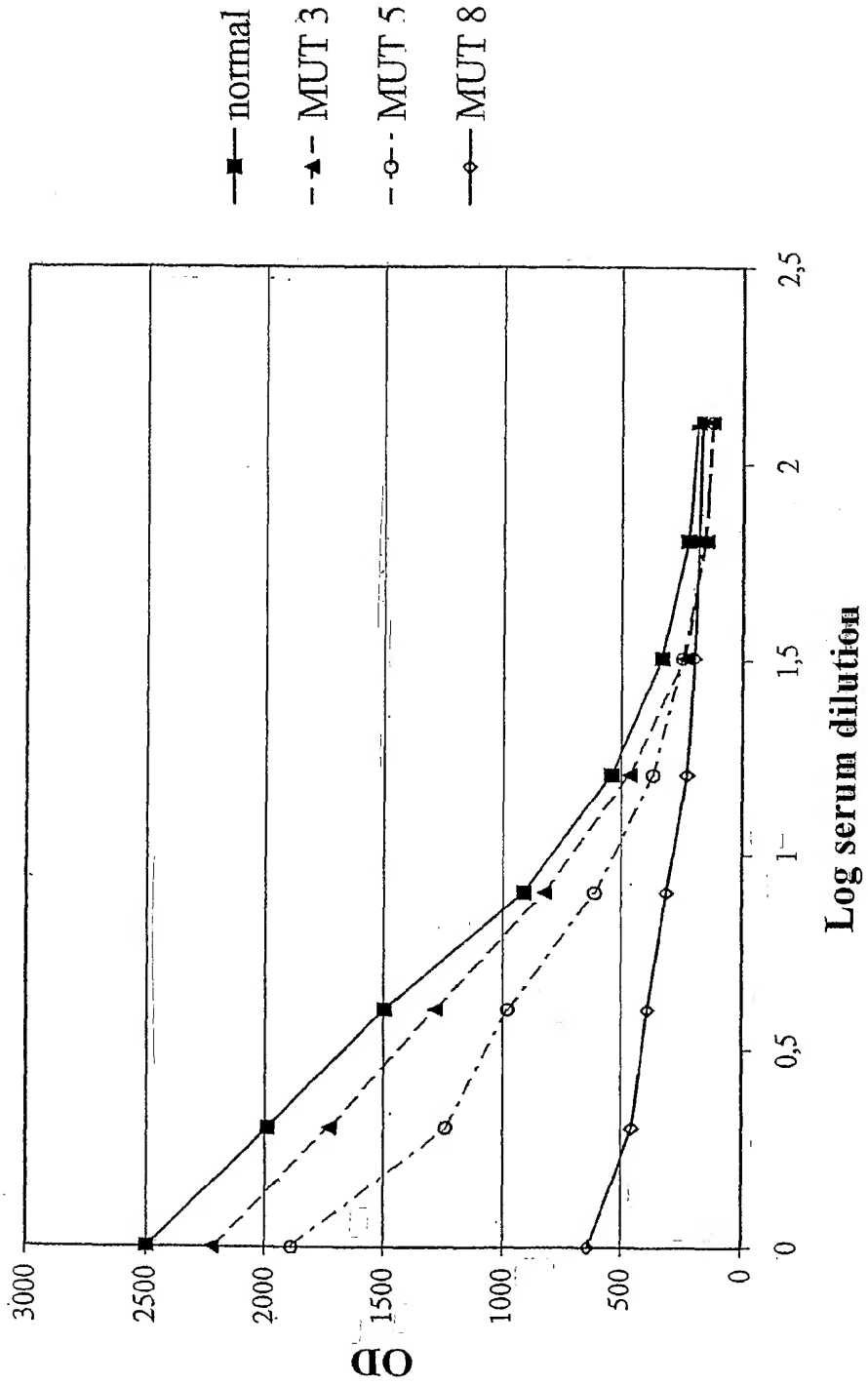
12. A composition as claimed in claim 11, in the form of a vaccine.

Figure 1



N: Par j 2 normal recombinant
5 : Par j 2 with 5 amino acidic modifications: Gln_{19}, Ala_{23}, Ala_{27}, Ala_{41}, Ala_{46}.
8 : Par j 2 with 8 amino acidic modifications: Gln_{19}, Ala_{23}, Ala_{27}, Gly_{35}, Ala_{41}, Ala_{46}, Gly_{73}, Ser_{78}.
1-14: single sera of subject allergic to Parietaria
Pool +: pool positive sera
Pool -: pool negative sera

Figure 2
Analysis of IgE reactivity of the variants of the Par j 2 allergen



SEQUENCE LISTING

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